**Vitamin D and Gynecologic Outcomes:
Insights from the EAGeR Trial**

Thesis submission by

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ABSTRACT

Context: Vitamin D is associated with a host of reproductive outcomes, but there is little research investigating these relationships in healthy, regularly cycling, premenopausal women.

Objective: Our objective was to assess the relationship between vitamin D and hormonal biomarkers, sporadic anovulation, menstrual cycle length, and premenstrual syndrome and its symptoms. We hypothesize that vitamin D will be inversely associated with aberrations in reproductive and gynecologic function.

Methods: This was a prospective cohort of 1191 participants attempting to conceive, aged 18-40, with 1-2 prior pregnancy losses, no history of infertility, and enrolled in the EAGeR trial. Patients answered questionnaires regarding demographic information and gynecologic histories and serum samples were collected pre-randomization. Patients collected and froze daily first-void urine samples for up to two menstrual cycles. Patients were followed for risk of anovulation for two menstrual cycles and followed all together for up to six menstrual cycles.

Results: Vitamin D was associated with free androgen index and sex hormone binding globulin concentration, but not total testosterone, free testosterone, or dehydroepiandrosterone sulfate. Vitamin D was negatively associated with estrone-1-glucuronide in urine but not with pregnanediol glucuronide. Vitamin D was not associated with menstrual cycle length or its phase components and was not associated with risk of sporadic anovulation. Vitamin D was associated with breast tenderness/fullness and generalized aches and pains during the premenstrual week but not with other symptoms or overall risk of PMS.

Conclusions: Vitamin D may play a role in a host of reproductive and endocrine outcomes, including the bioavailability of androgens, concentrations of estrogens, and physical symptoms of PMS.

INTRODUCTION

Vitamin D is well-known for its effects on bone health and metabolism. Vitamin D₂ and D₃ are hydroxylated by the liver to form 25-hydroxyvitamin D (25[OH]D) (1). This form is circulated in the bloodstream bound to a binding protein (2) and measured to clinically evaluate serum concentrations in humans (3). Further hydroxylation in the kidneys yields 1,25-dihydroxyvitamin D (calcitriol), which is the most biologically active (4). It binds to vitamin D receptors (VDR) in the intestines to then promote the absorption of calcium (5,6), promotes osteoclast resorption of bone, and is critical to normal parathyroid function (7). Yet vitamin D is involved in so much more than just calcium homeostasis. Vitamin D is also associated with a host of reproductive outcomes (8-10) and has receptors in the ovary, uterus, placenta, hypothalamus, and pituitary gland (11-13). The vitamin has been implicated in a number of pathologies affecting women, ranging from polycystic ovary syndrome (PCOS) to premenstrual syndrome (PMS), yet little is known about its function and relationships to various gynecologic and reproductive endpoints in healthy, regularly cycling women.

Vitamin D deficiency is defined by the Endocrine Society as serum 25(OH)D concentrations < 20.0 ng/mL, and insufficiency is defined as a concentration $\geq$ 20.0 ng/mL but < 30.0 ng/mL (14). NHANES data suggest that 46.1% of Americans are deficient in vitamin D (15). Among reproductive age women, vitamin D insufficiency has been reported in 26.8% of Quebecois women (16). Here in the United States, one study found that 2.0% and 44.9% of white and black pregnant women (4-21 weeks' gestation) were respectively vitamin D deficient while another 41.2% and 54.1% were respectively insufficient (17). Given the size of the population that does not have sufficient serum vitamin D concentrations, understanding all of its aberrant effects is critical for population health. Thus, the purpose of this work was to investigate vitamin D's

relationship with various gynecologic and endocrinologic outcomes in healthy, premenopausal women. We hypothesize that insufficient vitamin D status is associated with aberrations in reproductive and gynecologic function: specifically, androgens, menstrual cycle function, and premenstrual syndrome.

Vitamin D and Androgens

Understanding ovulatory function is essential to understanding premenopausal women's health. We became interested in understanding vitamin D's influence on androgens because polycystic ovary syndrome (PCOS) is simultaneously the most common endocrinopathy in women worldwide (18)—specifically causing hyperandrogenism specifically—and the most common cause of ovulatory dysfunction (19). One small study of normal-BMI PCOS patients and healthy, non-obese women demonstrated a positive relationship between vitamin D and total testosterone (TT) (20) while another similar study found no relationship (21). Results among postmenopausal women show a negative relationship between vitamin D and TT (22,23). Some studies have also investigated the bioavailability of testosterone. One study of 2931 postmenopausal women demonstrated a negative relationship between vitamin D and free testosterone (fT) (23). With regards to sex hormone binding globulin (SHBG)—which binds and limits the bioavailability of circulating free androgens and estrogens (24)—a positive relationship has been previously observed among both PCOS patients (25,26) and postmenopausal women (23,27).

There is also strong biological rationale for the association between vitamin D and the bioavailability of androgens mediated through SHBG. In particular, much prior work has focused on genetic variation in the vitamin D receptor (VDR), hyperandrogenism, and PCOS. Patients with at least one copy of the gene for VDR polymorphism *Apa-I* were more likely to have PCOS than

those with none; those who were homozygous for the polymorphism were most likely to have PCOS (28). In a different study, *Apa-I* was associated with higher testosterone and androstenedione concentrations than other VDR polymorphisms, though these were attenuated after adjustment for vitamin D, calcium, BMI, and month of blood draw (29). A study of 260 PCOS patients and 221 normo-ovulatory controls found that when compared to normal genotype GG (guanine-guanine) those with single nucleotide polymorphisms of either one or two adenines (GA or AA) had a higher severity of PCOS but were not at increased risk for the disease itself (30). Finally, a study of 56 PCOS patients revealed that the VDR *Bsm-I* polymorphism was associated with lower SHBG concentrations (31), which is especially interesting since PCOS itself is associated with diminished concentrations of SHBG (32). While other methods of biologic interaction between vitamin D, SHBG, and androgens are possible, it seems likely that VDR polymorphisms play a role in linking vitamin D and its receptor function to the androgen-related biochemical and clinical features classically observed in PCOS. Specifically, if vitamin D insufficiency does influence SHBG homeostasis, then total testosterone could remain unchanged while bioavailability would increase, therefore raising the potential for women to suffer from the physical effects of elevated androgens while having clinically “normal” total concentrations.

Exploring the normal physiologic relationships between vitamin D, SHBG, and androgens in healthy premenopausal women may help us understand this relationship more clearly since currently available data has only examined them in PCOS patients and postmenopausal women. Certainly, an improved understanding in the nonpathologic context may then inform our understanding of the pathophysiology of PCOS and shed light on future strategies to improve androgen excess. Therefore, we aimed to evaluate the relationship between vitamin D and androgen biomarkers in a large cohort of healthy, premenopausal women.

Vitamin D and Menstrual Cycle Function

Since the pituitary gland has vitamin D receptors (33), it is also plausible that vitamin D may affect ovulation, follicular dynamics, and menstrual hormone levels; however, the data is sparse. The largest study suggesting a link between vitamin D and estrogens in postmenopausal women was suggested by results from the Multi-Ethnic Study of Atherosclerosis (MESA) (23), but little work has been done in premenopausal women. An analysis of data from the Nurses' Health Study II found no relationship between vitamin D intake and anovulatory infertility among 18,555 women (34). One previous pilot study suggested that metformin and vitamin D synergistically increase dominant follicle counts (35), but a larger trial ultimately found no improvement in follicle maturation or menstrual irregularities and infertility with metformin plus calcium and vitamin D supplementation. (36). Given that 3-18% of menstrual cycles are sporadically anovulatory (37), and that anovulatory infertility is the most common cause of infertility (38), understanding the effect that vitamin D has on sporadic anovulation in healthy women is of critical importance to understanding the physiology of anovulation.

We know that menstrual cycle length is related to follicular phase estradiol concentrations, an earlier rise in follicle stimulating hormone, and increased risk of sporadic anovulation (39); however, there are large data gaps when it comes to vitamin D and menstrual cycle length, so we became particularly interested in this relationship as they may shed some light on the influence on hormonal dynamics. One study of African American women found vitamin D to be associated with reduced odds of long menstrual cycles, suggesting that vitamin D influences menstrual cycle length and may potentially play a role in ovarian function (40). Another study by the same research group found vitamin D levels to be associated with irregular menstrual cycles but with neither short nor long cycles (41). There is some data among PCOS patients to suggest that a relationship

between menstrual cycle function exists. A pilot study evaluating calcium plus vitamin D supplementation in PCOS patients found no improvement in cycle regularity in groups taking both or both plus metformin compared to patients taking metformin alone (42). Another study of 13 PCOS patients undergoing vitamin D repletion plus calcium therapy found that normal menstrual cyclicity returned in 7 of the 13 women, suggesting that calcium homeostasis may be responsible for arrested follicular development in women with PCOS (43). Yet these results are not necessarily generalizable to wider populations of women, as the studies were small and PCOS only affects approximately 15-20% of women (44). Furthermore, few studies utilize serum vitamin D concentrations, instead relying on vitamin D intake; thus, we set out to utilize this approach to examine the relationships between vitamin D and menstrual cycle hormones, sporadic anovulation, and menstrual cycle length in healthy, regularly cycling women.

Vitamin D and Premenstrual Syndrome

Premenstrual syndrome (PMS) affects approximately 48% of women (45) and is associated with mild to severe psychological and physical symptoms that typically occur during the luteal phase of the menstrual cycle. Symptoms can affect normal day-to-day life, including activities and interpersonal relationships (46). PMS symptoms include but are not limited to: irritability, anxiety and depression, mood swings, breast tenderness, headaches, and bloating (47,48).

Non-pharmaceutical therapies for PMS symptom relief, particularly related to dietary intake, are of great interest as they offer the potential for low-cost strategies to improve health and well-being. Hypocalcemia is known to cause symptoms that also occur in PMS, like muscle cramps, irritability, anxiety, and fatigue (49). Vitamin D has been proposed as an alternative because PMS is hypothesized to reflect a hypocalcemic state (50) and vitamin D plays a critical

role in calcium metabolism and homeostasis. Vitamin D has also been hypothesized to improve dysmenorrhea through a reduction in proinflammatory cytokines (51). 1,25-dihydroxyvitamin D lowers cyclooxygenase-2 expression in endometrial tissue, leading to declines in prostaglandins, which are essential for menstrual cycle function (52). Vitamin D has anti-inflammatory properties and has been shown to deter prostaglandin function which may also play a role (53). Understanding interactions between the two in healthy women is essential for future work, thus we aimed to evaluate the relationship between serum vitamin D concentrations and PMS symptoms in healthy, regularly cycling women.

METHODS

The EAGeR trial was a multicenter, block-randomized, double-blind, placebo controlled trial of daily low dose aspirin (LDA, 81 mg) in 1,228 women recruited from four U.S. medical centers from 2007 to 2011. Institutional Review Board approval was obtained at each study site and the data coordinating center. All participants provided written informed consent. The trial was registered with ClinicalTrials.gov, number NCT00467363. Full details of the study design, methods, and participant characteristics have been published elsewhere (54). The analyses presented here use the EAGeR trial cohort to answer important questions regarding the role of vitamin D on reproductive health outcomes.

Study Design and Population

Women trying to conceive who were 18-40 years old with regular menstrual cycles of 21-42 days in length, documented confirmation of 1 or 2 prior pregnancy losses, and up to two prior live births were eligible for the EAGeR trial (54). Exclusion criteria for all women included, but were not limited to: clinical indication for use of anticoagulant therapy or chronic use of non-steroidal anti-inflammatory drugs; bleeding disorders; presence of unstable mental disorders; major medical disorders (e.g. diabetes, hypertension, etc.); and any prior diagnosis of infertility or sub-fertility including related conditions such as PCOS, endometriosis, or pelvic inflammatory disease (54). Furthermore, all women must have been off long acting hormonal contraceptive medication (e.g. Depo-Provera, Norplant, intrauterine device) for at least 12 months or off oral contraceptive pills or other exogenous hormones (e.g. patch, ring) 3 months prior to enrollment.

Study Procedures

Participants attended a baseline study visit prior to randomization timed to occur around day 2–4 of their menstrual cycle. During this baseline visit, participants completed questionnaires of reproductive health status and demographic and lifestyle factors. In addition, anthropometric measures and blood samples were collected. Samples were centrifuged and serum and plasma were aliquoted and frozen within 90 minutes. Samples were stored at -80°C until analysis. Fertility monitors were provided to assist with timing of intercourse to optimize conception, evaluate ovulation status and scheduling subsequent study visits which were also timed to menstrual cycle phase (Clearblue Easy Fertility Monitor; Inverness Medical, Waltham, MA).

During the first two menstrual cycles of study participation, women collected daily first morning urine samples which were stored in a freezer and provided frozen to study staff at clinic visits, after which they were stored at -80°C until analysis. Women were followed up for up to six menstrual cycles.

Vitamin D Assessment

Preconception 25-hydroxyvitamin D (25(OH)D) concentrations were measured in serum collected at baseline using the 25(OH)D ELISA solid phase sandwich enzyme immunoassay (BioVendor R&D, Ashville, NC). The interassay laboratory coefficients of variation (CVs) were 15.8 and 13.1% at mean concentrations of 15.5 and 41.6 ng/mL, respectively, for lyophilized manufacturer's controls, and 17% for an in-house pooled serum control. All values were above the lower limit of detection of 1.6 ng/mL.

Outcome Measures

Androgens

Total testosterone concentration (TT, ng/dL) was determined by liquid chromatography and tandem mass spectrometry using a Shimadzu Prominence Liquid Chromatogram (Shimadzu Scientific Instruments, Inc., Columbia, MD) with an ABSceix 5500 tandem mass spectrometer (AB SCIEX, Framingham, MA). Interassay coefficients of variance (CVs) were 2.0% at 189.81 and 1.4% at 809.54 ng/mL. Free testosterone (fT) was calculated as $24.00314 \times TT / \log_{10} SHBG - 0.0499 \times TT^2$ and free androgen index (FAI) as $100 \times (TT / SHBG)$, where TT = total testosterone in nmol/L and SHBG = sex hormone binding globulin in nmol/L (55). SHBG concentration was determined by SHBG reagent/sandwich immunoassay method/electrochemiluminescence (Roche Diagnostics, Indianapolis, IN) utilizing a Roche COBAS 6000 chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Interassay CVs were 3.0% at 55.64 nmol/L and 3.8% at 19.74 nmol/L. Dehydroepiandrosterone sulfate (DHEAS) was determined by DHEA-S reagent/competitive immunoassay method/ electrochemiluminescence using a Roche COBAS 6000 chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Interassay CVs were 4.6% at 5.43 μ mol/L and 4.9% at 13.01 μ mol/L. Insulin was measured by sandwich immunoassay method using a Roche COBAS 6000 chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Interassay CVs were 3.1% at 121.2 pmol/L and 3.1% at 377.9 pmol/L.

Menstrual Cycle Function

Urinary measures were conducted at relevant menstrual cycle phases using daily first-morning urine collected at home during the first two cycles of study participation. If women became pregnant during the first cycle, only their first cycle of urine measures were used. Estrone-

3-glucuronide (E1G) and pregnanediol glucuronide (PdG) were determined by E1G/PdG Multiplex competitive chemiluminescence assay (Quansys Biosciences, Logan, UT). The interassay CVs for E1G were 16.9% and 20.2% at mean concentrations of 36.3 ng/mL and 1.9 ng/mL, respectively, for lyophilized manufacturer's controls and were 14.2% and 15.2% for in-house pooled urine controls at mean concentrations of 23.7 ng/mL and 10.2 ng/mL, respectively. The interassay CVs for PdG were 19.2% and 12.2% at mean concentrations of 1.986 μ g/mL and 0.1069 μ g/mL for lyophilized manufacturer's controls, respectively, and were 23.2% and 20.2% for in-house pooled urine controls at mean concentrations of 4.061 and 1.064 μ g/mL, respectively.

Menstrual cycles ending in pregnancy were considered ovulatory. Anovulation was assessed using urinary hormone concentrations and fertility monitor data from the first two cycles of study participation among women who did not achieve a pregnancy in the first two cycles and was based on the following step by step algorithm.

1. Cycles with maximum luteal phase PdG < 5 μ g/mL were considered anovulatory (n=259 of 1,353 cycles) (56).
2. For cycles without an available luteal-phase PdG concentration, the cycle was considered anovulatory if the fertility monitor LH concentration was < 2.5 times the average of the previous 5 days (n=37 of 147 cycles) (57).
3. Lastly, if adequate sequential LH data were unavailable from the fertility monitor, a *peak fertility* reading on the monitor was considered ovulatory (n=28) and all other cycles were unable to be classified (n=160) (58).

Cycle length was collected prospectively for all cycles of follow up (up to 6 prospective) and phase lengths were collected prospectively for two menstrual cycles. Cycle length was defined as the number of days between first days of menstrual bleeding. Follicular phase length was

calculated as the number of days between the first day of menstrual bleeding and the expected ovulation day based on LH peak on a fertility monitor, and luteal phase length was calculated as the number of days between LH peak on the monitor and the day prior to onset of next menses.

Premenstrual Syndrome

Participants completed a questionnaire at baseline detailing five psychological symptoms (depression or sadness, tension or irritability, anxiety or nervousness, anger or aggression or short temper, and crying spells), and nine physical symptoms (swelling of hands or feet, breast tenderness or fullness, abdominal bloating, lower abdominal cramping, generalized aches and pains, lower backache, headache, fatigue, and insomnia) that were associated with PMS over the past 12 months. They rated their symptoms in the past 12 months during a typical week leading up to menses on a scale of: 0=none, 1=mild, 2=moderate, or 3=severe. A total symptom severity score was calculated by adding the severity ratings for all symptoms (total possible score 42). Scores were calculated for the week prior to menses and the week after menses for comparison and assessment of PMS. Participants were then denoted as either having moderate/severe or mild/none for each symptom during the premenstrual week. The total number of moderate/severe symptoms during the premenstrual week was calculated overall and by symptom type.

Two different sets of criteria were used to define PMS:

1. Women with ≥ 3 moderate to severe symptoms during premenstrual week with a minimum of 1 being psychological, and a total symptom severity score during the premenstrual week at least 30% greater than the total symptom severity score during the postmenstrual week, were categorized as having PMS. This set of criteria was adapted from Borenstein et al. and used in other studies as well (59-61).

2. Women with ≥ 5 moderate to severe symptoms during premenstrual week, with at least 2 being psychological, and a total symptom severity score during the premenstrual week at least 30% greater than the total symptom severity score during the postmenstrual week, were categorized as having PMS. This set of criteria reflects more severe premenstrual symptoms as used by Gollenberg et al. (61).

Statistical Analysis

Participants were determined to be vitamin D-sufficient ($25(\text{OH})\text{D} \geq 30.0 \text{ ng/mL}$) or vitamin D-insufficient ($25(\text{OH})\text{D} < 30.0 \text{ ng/mL}$) using baseline $25(\text{OH})\text{D}$ serum concentrations (14). Demographic and reproductive histories at baseline were compared by vitamin D status using student's t-tests and chi-square tests where appropriate. SAS version 9.4 (SAS Institute, Cary, North Carolina) was used for all statistical analysis.

Androgens

Generalized linear regression was used to estimate the associations between baseline vitamin D and TT, fT, FAI, DHEAS, and SHBG. All hormonal markers were natural log-transformed to achieve normal distributions, and thus results are reported as percent change in the dependent variable per unit change in independent variable with 95% CI (62). Models were adjusted for age, BMI, current smoking status, race, income, physical activity, and season of blood draw. Models for SHBG were additionally adjusted for TT, E1G, and insulin, as they are known to affect SHBG concentrations (63). Androgen models were not adjusted for treatment arm as the serum was collected prior to randomization.

Menstrual Cycle Function

Linear mixed models were used to prospectively evaluate associations between vitamin D and follicular phase E1G and luteal phase PdG accounting for multiple measures per woman over both cycles of follow up. Both E1G and PdG were natural log-transformed to achieve normal distributions, and thus results are reported as percent change in the dependent variable per unit change in independent variable with 95% CI (62). Models were adjusted for age, BMI, current smoking status, race, income, physical activity, season of blood draw, and treatment arm.

Log-binomial regression was used to prospectively estimate the risk ratios (RR) and 95% confidence intervals (CI) for sporadic anovulation during the first two cycles of study participation, taking into account multiple cycles per woman. Models were adjusted for age, BMI, current smoking status, race, income, physical activity, season of blood draw, and treatment arm.

Linear mixed models were used to prospectively evaluate the relationships between vitamin D and follicular phase, and luteal phase lengths across 2 prospective cycles of follow up. Linear mixed models were also used to evaluate the relationship between total cycle length and vitamin D for up to 6 cycles of follow up and were weighted to account for number of cycles each participant contributed. Cycles ending in pregnancy were excluded from the analysis. Models were adjusted for age, BMI, current smoking status, race, income, physical activity, season of blood draw, and treatment arm.

Premenstrual Syndrome

Log-binomial regression was used to estimate the RR and 95% CI for both PMS criteria and for the individual symptoms of moderate/severe intensity during the premenstrual week. Logistic regression was used estimate the odds ratio (OR) and 95% CI for swelling of the hands

and feet due to lack of power. Generalized linear regression was used to calculate the β -coefficient and 95% CI for the association between vitamin D and total number of moderate/severe symptoms, total number of moderate/severe psychological symptoms, and total number of moderate/severe physical symptoms. All models were adjusted for age, BMI, current smoking status, race, income, physical activity, season of blood draw. Models were not adjusted for treatment arm as the questionnaires were filled out and serum collected prior to randomization.

RESULTS

Vitamin D data was available for 1191 patients, for whom the demographic characteristics and gynecologic histories are provided in Table 1. Of the 1191 patients, 555 had sufficient and 636 had insufficient serum vitamin D concentrations. A higher proportion of women with sufficient vitamin D were white than vitamin D-insufficient women. Women with sufficient vitamin D also had lower BMI and smaller waist circumference, sum of skinfolds, and central-to-peripheral skinfold ratio than those with insufficient concentrations. There were no differences between both groups' reported gynecologic histories.

Androgens

Vitamin D was not associated with TT and DHEAS (Table 2). However, vitamin D was negatively associated with fT in the unadjusted model, but this relationship was attenuated after adjustment for potential confounders. Vitamin D concentration was negatively associated with FAI. Vitamin D concentration was positively associated with SHBG concentration. These findings remained consistent after additional adjustment for TT, E1G and insulin.

Menstrual Cycle Function

There was a negative relationship between E1G and vitamin D but no relationship between PdG and vitamin D (Table 2).

14.6% of participants experienced a sporadic anovulatory cycle in the first two cycles of follow up, with 13.1% of vitamin D sufficient and 15.1% of insufficient patients experiencing one. Vitamin D was not associated with the risk of sporadic anovulation (Table 3).

The mean menstrual cycle length was 30.1 days and 31.3 days for vitamin D sufficient and insufficient women, respectively. The respective mean follicular phase lengths for sufficient and insufficient groups were 15.3 and 15.4, and the luteal phase length means were 13.4 and 13.2 days, respectively. There were no significant relationships between vitamin D and menstrual cycle length, follicular phase length, or luteal phase length in the continuous vitamin D models. Vitamin D insufficiency was associated with menstrual cycle length but the results were attenuated after adjustment for confounders (Table 4).

Premenstrual Syndrome

Vitamin D concentrations and PMS data were available for 1139 participants. 39.2% of women with sufficient vitamin D and 41.9% of women with insufficient vitamin D met the first set of criteria for PMS, while 23.4% and 24.8% respectively met the second criteria. The most common physical symptom reported was lower abdominal cramping, which was experienced by 37% of women, followed by abdominal bloating (34.1%) and fatigue (30.8%). The most common psychological PMS symptom reported was tension or irritability (40.6%), followed by anger, aggression, and short temper (28.6%). Vitamin D was not associated with risk of PMS based on either of the two sets of criteria. Vitamin D was associated with the total number of premenstrual symptoms, but the relationship was attenuated after adjustment in both categorical and continuous models (Table 5).

Vitamin D insufficiency was associated with number of moderate/severe physical symptoms, though results in the continuous models were attenuated after adjustment. Vitamin D was associated with increased risk of breast tenderness or fullness and generalized aches and pains. There was no relationship between vitamin D and abdominal bloating, cramping, and fatigue,

swelling of the hands and feet, headache or insomnia after adjustment for confounders (Table 6).

There were no significant relationships with vitamin D and either number of psychological symptoms or individual psychological symptoms of PMS (Table 7).

TABLES

Table 1. Characteristics of women in the EAGeR trial by vitamin D status at baseline.

	Vitamin D Status		p-value
	Vitamin D Sufficient (≥ 30 ng/mL)	Vitamin D Insufficient (< 30 ng/mL)	
	N=555	N=636	
Age, mean $\pm$ SD (years)	28.9 $\pm$ 4.8	28.6 $\pm$ 4.8	0.36
BMI, mean $\pm$ SD (kg/m²)	24.5 $\pm$ 5.06	27.8 $\pm$ 7.3	<0.0001
Waist circumference, mean $\pm$ SD (cm)	82.7 $\pm$ 12.3	90.5 $\pm$ 16.6	<0.0001
Sum of skinfolds, mean $\pm$ SD (mm)	92.0 $\pm$ 27.6	108.2 $\pm$ 35.1	<0.0001
Central to peripheral skinfold ratio, mean $\pm$ SD	0.7 $\pm$ 0.2	0.8 $\pm$ 0.2	<0.0001
Race, n (%)			<0.0001
White	542 (98%)	586 (92%)	
Non-White	13 (2%)	50 (8%)	
Married or living with partner, n (%)	542 (98%)	620 (97%)	0.85
Education > high school, n (%)	491 (89%)	542 (85%)	0.08
Income, n (%)			0.0004
$\geq$ \$100,000	209 (38%)	261 (41%)	
\$75,000-\$99,999	84 (15%)	63 (10%)	
\$40,000-\$74,999	99 (18%)	75 (12%)	
\$20,000-\$39,999	128 (23%)	179 (28%)	
$\leq$ \$19,999	35 (6%)	57 (9%)	
Current smokers, n (%)	69 (13%)	79 (13%)	0.99
Parity, n (%)			0.09
Nulliparous	271 (49%)	279 (44%)	
Parous (1 or 2 prior live births)	284 (51%)	357 (56%)	
Number of previous pregnancy losses, n (%)			0.84
1	211 (33%)	425 (67%)	
2	181 (33%)	374 (67%)	
Ever used hormonal contraception/meds, any reason, n (%)	435 (82)	472 (79%)	0.24
Years for periods to become regular	1.5 (4.2%)	1.7 (4.3%)	0.4824
Usual menstrual bleeding, d	5.0 (1.3%)	5.1 (2.1%)	0.5192
Number of periods in past 6 months	4.6 (1.6%)	4.6 (1.6%)	0.376
Regular periods, n (%)			0.0628
Yes	439 (82%)	500 (84%)	
No	77 (14%)	61 (10%)	
Don't Know	22 (4%)	34 (6%)	
Age at menarche, years	12.8 (1.5)	12.7 (1.5)	0.21
Aspirin treatment assignment, n (%)	285 (51%)	313 (49%)	0.46

SD = standard deviation; n = number

Table 2. Associations between vitamin D and androgen concentrations and other endocrine characteristics among women in the EAGeR trial.

	Vitamin D Sufficient (≥ 30 ng/mL)	Vitamin D Insufficient (< 30 ng/mL)	Vitamin D (per 10 ng/mL)
<u>Serum Measurements</u>			
Total testosterone (TT)			
Mean $\pm$ SD (ng/dL)	22.1 $\pm$ 12.0	22.5 $\pm$ 9.7	
Unadjusted percent change (95% CI)	Reference	3% (-2%, 8%)	-2% (-4%, 0.2%)
Adjusted percent change (95% CI)	Reference	0% (-5%, 5%)	0% (-2%, 2%)
Free testosterone (fT)			
Mean $\pm$ SD (ng/dL)	0.3 $\pm$ 0.2	0.3 $\pm$ 0.1	
Unadjusted percent change (95% CI)	Reference	9% (4%, 13%)	-4% (-6%, -2%)
Adjusted percent change (95% CI)	Reference	2% (-2, 7)	-2% (-4%, 0.4%)
Free Androgen Index (FAI)			
Mean $\pm$ SD	1.2 $\pm$ 0.9	1.6 $\pm$ 1.2	
Unadjusted percent change (95% CI)	Reference	25% (18%, 31%)	-11% (-14%, -8%)
Adjusted percent change (95% CI)	Reference	10% (3%, 16%)	-5% (-8%, -2%)
DHEAS			
Mean $\pm$ SD (μ mol/L)	4.8 $\pm$ 2.2	5.1 $\pm$ 2.6	
Unadjusted percent change (95% CI)	Reference	4% (-2, 10)	-2% (-4%, 0.5%)
Adjusted percent change (95% CI)	Reference	1% (-5%, 7%)	-1% (-3%, 2%)
SHBG			
Mean $\pm$ SD (nmol/L)	75.1 $\pm$ 36.1	61.2 $\pm$ 31.4	
Unadjusted percent change (95% CI)	Reference	-21% (-27%, -16%)	9% (7%, 11%)
Adjusted percent change (95% CI)	Reference	-10% (-15%, -4%)	4% (2%, 7%)
Additional adjustment for insulin, TT, and E1G	Reference	-10% (-16%, -4%)	7% (4%, 10%)
<u>Urinary hormones</u>			
Estrone-3-Glucoronide (E1G), follicular phase			
Mean $\pm$ SD (ng/mL)	30.5 $\pm$ 26.3	33.2 $\pm$ 34.1	
Unadjusted percent change (95% CI)	Reference	6% (-4%, 17%)	-7% (-12%, -2%)
Adjusted percent change (95% CI)	Reference	2% (-9%, 13%)	-5% (-10%, -0.2%)
Pregnanediol-3-Glucoronide (PdG), luteal phase			
Mean $\pm$ SD (μ g/mL)	20.7 $\pm$ 30.5	23.3 $\pm$ 114.1	
Unadjusted percent change (95% CI)	Reference	-5% (-17%, 6%)	-2% (-7%, 3%)
Adjusted percent change (95% CI)	Reference	-5% (-17%, 7%)	-2% (-7%, 4%)

SD = standard deviation; CI = confidence interval.

Bold results are statistically significant at $\alpha=0.05$.

Adjusted models control for age, body mass index, smoking status, race, income, exercise, season of blood draw.

Adjusted E1G and PdG models also control for treatment arm as those data were collected prospectively after randomization.

Table 3. Association between vitamin D and sporadic anovulation among women in the EAGeR trial.

	Vitamin D Sufficient (≥ 30 ng/mL)	Vitamin D Insufficient (< 30 ng/mL)	Vitamin D (per 10 ng/mL)
Incidence of sporadic anovulation	13.1%	15.1%	
Unadjusted RR (95% CI)	Reference	1.23 (0.97, 1.56)	0.97 (0.87, 1.08)
Adjusted RR (95% CI)	Reference	1.04 (0.81, 1.34)	1.04 (0.94, 1.15)

RR = risk ratio; CI = confidence interval.

Bold results are significant at $\alpha=0.05$.

Adjusted models control for age, body mass index, smoking status, race, income, exercise, season of blood draw, and treatment arm.

Table 4. Associations between vitamin D and menstrual cycle and phase lengths

	Vitamin D Sufficient (≥ 30 ng/mL)	Vitamin D Insufficient (< 30 ng/mL)	Vitamin D (per 10 ng/mL)
Cycle length			
Mean $\pm$ SD (days)	30.1 $\pm$ 6.6	31.3 $\pm$ 10.5	
Unadjusted, β (95% CI)	Reference	0.88 (0.21, 1.54)	-0.19 (-0.46, 0.09)
Adjusted, β (95% CI)	Reference	0.59 (-0.10, 1.28)	-0.03 (-0.32, 0.26)
Follicular phase length			
Mean $\pm$ SD (days)	15.3 $\pm$ 2.7	15.4 $\pm$ 2.8	
Unadjusted, β (95% CI)	Reference	0.14 (-0.18, 0.38)	0.03 (-0.08, 0.16)
Adjusted, β (95% CI)	Reference	0.03 (-0.27, 0.32)	0.06 (-0.07, 0.19)
Luteal phase length			
Mean $\pm$ SD (days)	13.4 $\pm$ 4.7	13.2 $\pm$ 4.4	
Unadjusted, β (95% CI)	Reference	-0.21 (-0.67, 0.25)	-0.07 (-0.26, 0.13)
Adjusted, β (95% CI)	Reference	-0.23 (-0.71, 0.25)	-0.04 (-0.25, 0.17)

SD = standard deviation; CI = confidence interval.

Bold results are significant at $\alpha=0.05$.

Adjusted models control for age, BMI, smoking status, race, income, exercise, season of blood draw, and treatment arm.

Table 5. Associations between vitamin D and premenstrual syndrome

	Vitamin D Sufficient (≥ 30 ng/mL)	Vitamin D Insufficient (≤ 30 ng/mL)	Vitamin D (per 10 ng/mL)
PMS Criteria 1			
Prevalence	39.2%	41.9%	
Unadjusted RR (95% CI)	Ref	1.07 (0.93, 1.23)	1.00 (0.93, 1.06)
Adjusted RR (95% CI)	Ref	1.02 (0.88, 1.18)	1.02 (0.96, 1.09)
PMS Criteria 2			
Prevalence	23.4%	24.8%	
Unadjusted RR (95% CI)	Ref	1.06 (0.86, 1.30)	0.96 (0.88, 1.05)
Adjusted RR (95% CI)	Ref	0.94 (0.76, 1.17)	1.00 (0.92, 1.10)
Number of Moderate/Severe Premenstrual Symptoms			
Mean + SD	3.0 $\pm$ 2.9	3.5 $\pm$ 3.2	
Unadjusted β (95% CI)	Ref	0.49 (0.13, 0.85)	-0.16 (-0.31, -0.01)
Adjusted β (95% CI)	Ref	0.32 (-0.05, 0.70)	-0.08 (-0.24, 0.07)

RR = risk ratio; CI = confidence interval; SD = standard deviation

Bold results are significant at $\alpha=0.05$

Adjusted models control for age, BMI, smoking status, race, income, exercise, season of blood draw

PMS criteria 1: ≥ 3 moderate to severe symptoms during premenstrual week (minimum of 1 psychological), and a total of symptom severity during premenstrual week at least 30% greater than the total score during the postmenstrual week (59-61).

PMS criteria 2: ≥ 5 moderate to severe symptoms during premenstrual week (at least 2 psychological), and a total of symptom severity score during premenstrual week at least 30% greater than the total score during the postmenstrual week (61).

Table 6. Associations between vitamin D and physical symptoms of PMS

	Vitamin D Sufficient (≥ 30 ng/mL)	Vitamin D Insufficient (< 30 ng/mL)	Vitamin D (per 10 ng/mL)
Number of Moderate/Severe Physical Symptoms			
Mean + SD	1.9 $\pm$ 2.0	2.3 $\pm$ 2.3	
Unadjusted β (95% CI)	Ref	0.42 (0.17, 0.67)	-0.15 (-0.25, -0.05)
Adjusted β (95% CI)	Ref	0.31 (0.06, 0.51)	-0.10 (-0.20, 0.01)
Swelling of Hands or Feet			
Prevalence	3.1%	5.4%	
Unadjusted OR* (95% CI)	Ref	1.75 (0.96, 3.18)	0.76 (0.56, 1.02)
Adjusted OR* (95% CI)	Ref	1.67 (0.87, 3.23)	0.78 (0.56, 1.08)
Breast Tenderness or Fullness			
Prevalence	23.5%	31.2%	
Unadjusted RR (95% CI)	Ref	1.33 (1.10, 1.61)	0.87 (0.79, 0.95)
Adjusted RR (95% CI)	Ref	1.27 (1.03, 1.55)	0.89 (0.81, 0.98)
Abdominal Bloating			
Prevalence	31.2%	36.6%	
Unadjusted RR (95% CI)	Ref	1.17 (0.995, 1.38)	0.94 (0.88, 1.01)
Adjusted RR (95% CI)	Ref	1.17 (0.98, 1.39)	0.95 (0.88, 1.03)
Lower Abdominal Cramping			
Prevalence	32.9%	40.3%	
Unadjusted RR (95% CI)	Ref	1.22 (1.05, 1.43)	0.93 (0.86, 1.001)
Adjusted RR (95% CI)	Ref	1.13 (0.96, 1.33)	0.97 (0.90, 1.04)
Generalized Aches and Pains			
Prevalence	13.5%	20.4%	
Unadjusted RR (95% CI)	Ref	1.52 (1.16, 1.98)	0.81 (0.71, 0.93)
Adjusted RR (95% CI)	Ref	1.33 (1.005, 1.78)	0.87 (0.76, 0.99)
Lower Backache			
Prevalence	20.7%	26.3%	
Unadjusted RR (95% CI)	Ref	1.27 (1.03, 1.57)	0.95 (0.87, 1.05)
Adjusted RR (95% CI)	Ref	1.19 (0.95, 1.48)	0.99 (0.90, 1.09)
Headache			
Prevalence	26.0%	25.8%	
Unadjusted RR (95% CI)	Ref	0.99 (0.81, 1.21)	0.97 (0.89, 1.06)
Adjusted RR (95% CI)	Ref	0.94 (0.76, 1.16)	1.00 (0.92, 1.09)
Fatigue			
Prevalence	28.1%	33.2%	
Unadjusted RR (95% CI)	Ref	1.18 (0.99, 1.41)	0.93 (0.85, 1.01)
Adjusted RR (95% CI)	Ref	1.09 (0.90, 1.31)	0.97 (0.89, 1.05)
Insomnia			
Prevalence	8.5%	10.5%	
Unadjusted RR (95% CI)	Ref	1.24 (0.86, 1.78)	0.93 (0.79, 1.10)
Adjusted RR (95% CI)	Ref	1.14 (0.78, 1.67)	0.97 (0.83, 1.15)

SD = standard deviation; RR = risk ratio

Bold results are significant at $\alpha=0.05$

Adjusted models control for age, BMI, smoking status, race, income, exercise, season of blood draw.

*Logistic regression was used to odds ratios (OR) due to lack of power.

Table 7. Associations between vitamin D and psychological symptoms of PMS.

	Vitamin D Sufficient (≥ 30 ng/mL)	Vitamin D Insufficient (< 30 ng/mL)	Vitamin D (per 10 ng/mL)
Number of Moderate/Severe Psychological Symptoms			
Mean + SD	1.2 $\pm$ 1.5	1.2 $\pm$ 1.5	
Unadjusted β (95% CI)	Ref	0.07 (-0.11, 0.25)	-0.01 (-0.08, 0.06)
Adjusted β (95% CI)	Ref	0.02 (-0.17, 0.21)	0.01 (-0.07, 0.09)
Depression or Sadness			
Prevalence	16.8%	17.9%	
Unadjusted RR (95% CI)	Ref	1.06 (0.83, 1.37)	1.01 (0.92, 1.12)
Adjusted RR (95% CI)	Ref	1.07 (0.82, 1.40)	0.99 (0.90, 1.12)
Tension or Irritability			
Prevalence	39.8%	41.3%	
Unadjusted RR (95% CI)	Ref	1.04 (0.90, 1.20)	0.99 (0.93, 1.05)
Adjusted RR (95% CI)	Ref	1.00 (0.86, 1.16)	1.00 (0.94, 1.07)
Anxiety or Nervousness			
Prevalence	17.9%	19.4%	
Unadjusted RR (95% CI)	Ref	1.09 (0.85, 1.38)	1.03 (0.93, 1.14)
Adjusted RR (95% CI)	Ref	1.06 (0.82, 1.36)	1.04 (0.94, 1.16)
Anger, Aggression, Short Temper			
Prevalence	28.5%	28.6%	
Unadjusted RR (95% CI)	Ref	1.00 (0.84, 1.21)	0.96 (0.89, 1.04)
Adjusted RR (95% CI)	Ref	0.93 (0.77, 1.13)	1.00 (0.92, 1.08)
Crying Spells			
Prevalence	13.9%	16.8%	
Unadjusted RR (95% CI)	Ref	1.21 (0.92, 1.60)	0.96 (0.85, 1.09)
Adjusted RR (95% CI)	Ref	1.11 (0.83, 1.48)	1.00 (0.88, 1.13)

SD = standard deviation; RR = relative risk; CI = confidence interval.

Bold results are significant at $\alpha=0.05$.

Adjusted models control for age, BMI, smoking status, race, income, exercise, season of blood draw, and treatment arm.

DISCUSSION

Androgens

Our results show increasing vitamin D concentrations were negatively associated with FAI and positively associated with SHBG in this cohort of healthy, premenopausal women with no diagnosis of infertility or PCOS. The positive relationship between vitamin D and SHBG is likely driving the negative relationship we observed with FAI, since FAI is calculated using serum concentration of SHBG. These results help elucidate the role vitamin D may play in the bioavailability of androgens, a potentially important factor in the pathophysiology of hyperandrogenism and its related complications for women of reproductive age. It is possible that women with biochemically “normal” total testosterone levels could still suffer from [mild] aberrant effects of testosterone if it is more bioavailable due to suboptimal vitamin D concentrations. However, it is important to note that our data regarding vitamin D and androgens is cross-sectional, so no conclusions as to how changes in vitamin D affect the bioavailability of androgens over time can be drawn. Also, we do not have information on phenotypic features related to androgen activity such as hirsutism or acne, thus we can only evaluate androgens biochemically and not clinically in this population.

Menstrual Cycle Function

Our results also show a negative relationship between vitamin D and E1G while showing a null relationship with PdG. We found no relationship between vitamin D and the risk of sporadic anovulation, menstrual cycle length, follicular phase length, and luteal phase length. It thus does not appear that vitamin D affects estrogen concentrations via ovarian follicular dynamics, as one would expect the other outcomes to differ in concert with estrogen if this were the case. If vitamin

D does in fact drive estrogen concentrations, it could explain how it affects the bioavailability of androgens, since estrogen increases SHBG production which in turns limits bioavailability. An important limiting factor to keep in mind when reading these results is our lack of serum estradiol data. However, we believe the prospectively collected E1G from daily urine samples compensates for this shortcoming as it is directly related to estradiol concentrations and is stable in urine (64).

Premenstrual Syndrome

We found no relationship between vitamin D and overall risk of PMS. We did find a relationship between vitamin D and both breast fullness/tenderness and generalized aches and pains in the premenstrual week, which in turn drove a relationship with number of moderate/severe physical symptoms of PMS. No significant relationships were found between vitamin D and psychological symptoms of PMS.

The biological mechanism underlying the relationship between vitamin D and generalized aches and pains in PMS may be better understood by investigating diseases with symptomatology similar to those caused by hypocalcemic states. One such is aromatase inhibitor musculoskeletal symptoms (AIMSS), characterized by a cluster of musculoskeletal symptoms that include bone, muscle, and joint pain; muscle stiffness; carpal tunnel syndrome; tenosynovitis; and allodynia (62,65-70), which are also symptoms of hypocalcemic diseases like vitamin D deficiency (70-72). Given that vitamin D is used to treat AIMSS (70,73-78), there may be similar pathophysiologic mechanisms between AIMSS and generalized aches and pain in PMS. Adult dorsal root ganglia in female rats have vitamin D receptors and enzymes whose expression changes when the rats undergo oophorectomy, indicating that ovarian hormones affect expression in sensory neurons in

these female rats (79). A similar mechanism may lead to premenstrual aches and pains modulated by [lack of] vitamin D.

It is important to note that our data is limited in that it compares vitamin D concentrations with the prevalence of PMS symptoms that were retrospectively reported over the past year. Data that prospectively compares the two might be able to more definitively establish a relationship between vitamin D and PMS symptoms.

Overall Strengths and Limitations

Overall, this was a large study among women with proven fecundity, regular cycles, and a history of prior pregnancy loss, expanding upon prior work in college-age populations and among a cohort of nurses. Further, our use of serum concentrations of vitamin D is a strength, as dietary assessments are unable to fully capture vitamin D status, and serum concentrations have been shown to be an accurate biological reflection of vitamin D status regardless of source (80,81). It should be noted that over 90% of participants in EAGeR were taking vitamin supplements, and it is possible that some women were consuming supplemental vitamin D. Though we do not have data on specific vitamin formulation, our serum assessment of vitamin D should overcome this limitation. Also, all of the women in the trial were fecund and had a history of 1-2 pregnancy losses, so it might not be generalizable to all reproductive age women. Notwithstanding, a large proportion of reproductive age women have had a pregnancy loss, so implications of these results are yet nonetheless broad (82).

CONCLUSIONS

In summary, we found relationships between serum vitamin D concentrations and SHBG, the bioavailability of androgens, and physical symptoms of PMS—specifically breast tenderness/fullness and generalized aches and pains—using data from the EAGeR trial. We also did not find a relationship between vitamin D and sporadic anovulation, menstrual cycle length, and psychological symptoms of PMS or overall risk of PMS, which is reassuring. To our knowledge, these are the first investigations into the relationships between vitamin D and androgens, anovulation, and menstrual length among healthy, regularly cycling, premenopausal women. These findings shed insights into normal physiology and may help us understand pathophysiologic processes plaguing many women worldwide.

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